Airway epithelium in obliterative airway disease
Qu, Ning

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Chapter 6

General Discussion
Heterotopic tracheal allografts in small rodents have been shown to share histopathological characteristics with human OB (1) and therefore provide a suitable animal model for the study of OB (2;3), although the pathogenesis may differ. Allografts in small rodents develop obliterative airway disease (OAD) within 3-4 weeks after transplantation (4-6). Histopathological analysis of these grafts reveals an early epithelium loss followed by fibroblast overgrowth; the overgrowing fibroblasts occlude the airway lumen. Human OB and animal OAD are both characterized histologically by airway epithelium damage and fibrotic luminal obliteration.

To study the airway epithelium and its behavior in OAD after transplantation, we used different animal models. In one part of the study the trachea transplantation model was used in rats (chapter 2), in other parts in mice (chapters 3, 4, and 5). These trachea transplant models facilitate the examination of the pathogenesis of the disease and the elucidation of the cellular and molecular mechanisms involved in its development. The model provides a less technically demanding alternative to whole lung transplantation in small rodents and may lead to quicker identification of new treatments that might prevent the development of post-transplantation OB in patients.

The experiments described in this thesis address several aspects of the role of airway epithelial cells and its behavior in OAD. These aspects are 1) the role of epithelium injury in OAD occurrence during the post-transplant period (chapter 2), 2) the role of epithelium specific immune responses in this injury (chapters 3 and 4) and 3) a possible therapeutic option through the application of C45RB antibodies for OAD prevention (chapter 5).

**Airway epithelium plays important role inhibiting OAD**

Our work supports the idea that airway epithelium is the main target tissue during alloimmune rejection and plays a crucial role in development of OAD (7-10). In chapter 2, results are presented upon transplantation of allogenic trachea grafts, which are in line with other studies of animal OAD models (3;6;11). Severe loss of epithelium was found to start at the first week after transplantation with total loss within 3 weeks. Full development of OAD was present at 4 weeks. It is interesting to note that the fibroproliferation started at week 2 after transplantation, secondary to airway epithelium injury and ultimate loss (chapter 2) as a result of acute rejection in our rat trachea
transplants. Furthermore, a direct effect of epithelial cells on fibroproliferation was demonstrated by grafting tracheas without epithelium, so-called denuded tracheas (chapter 2). In denuded allografts, immediate fibroproliferation was observed after grafting and fibroproliferation started within 6 days. Also in denuded isografts, which are not exposed to acute rejection, immediate fibroproliferation was observed after grafting. Interestingly, after partial denudation of isografts, the fibroproliferation stopped at the time point when epithelium started to recover (day 10). In addition, early fibroproliferation could be inhibited in denuded isografts by growth of seeded syngeneic epithelial cells. These findings indicate that the absence of epithelium results in fibroproliferation and that OAD is not the result from transplant rejection only, but could also result from other epithelium-targeted tissue injuries. It is likely that this is a reflection of a general tissue ‘injury-repair’ process in which epithelial cells are the injured targets and fibroblasts are the repair substitutes if epithelial cells are not able to regenerate and cope with the damage (12;13).

The interaction between epithelial cells and fibroblasts has been investigated in several studies (14). These indicate that epithelial cells release various cytokines which are involved in ‘epithelial cell-fibroblast’ interaction, such as fibrogenic growth factor (FGF) (15), prostaglandin E2 (PGE2) and transforming growth factor (TGF) beta (14;16). The FGF is known to induce apoptosis in epithelial cells and to stimulate fibroproliferation (15), whereas PGE2 and TGF-beta are the inhibitor and stimulator of fibroproliferation, respectively (14;17-19).

During the process of epithelial cell injury, for example during transplant rejection or chemical denudation, epithelial cells start to release FGF before they get into apoptosis (15). As this process continues, decreasing levels of PGE2 and increasing level of TGF-beta are released by the epithelial cells, giving fibroblasts the opportunity to grow. As a result, fibroblasts will replace the lost epithelium and, in addition, will grow into the lumen in an unregulated manner and finally obliterate the lumen as a consequence. From such a concept it can be put forward that epithelial cells are the main mediator in maintaining the airway architecture after transplantation. Presence of epithelial cells largely inhibits the fibroproliferation while OAD occurs as a result of an unbalanced situation. Thus, epithelium loss as a result of transplant rejection will initiate fibroproliferation and will eventually result into luminal obliteration.

Immune responses are a major component in the transplant rejection process. It is well documented that alloantigen specific antibodies (IgM and IgG), and
more importantly, immune cells (mainly CD4+, CD8+ T cells) contribute to the initiation and execution of destruction in lung transplantation (20-22) and trachea transplantation (23-25)

**Antibody responses in OAD**

Alloantibodies against donor tissue may be directly involved in OB and OAD. Clinical investigations in lung transplantation show that both anti-HLA class I and II antibodies are associated with epithelium damage in patients with OB (20;26-28). In an animal study antibody-mediated complement activation has been shown to induce epithelium injury and to contribute to the development of OAD in rat tracheal allografts. (29). These results are in line with our findings in chapter 3, that alloantibodies appear as early as one week after transplantation in recipients of fully MHC mismatched tracheas. In these studies immune responses towards tissues other than epithelium could not be excluded to play a role in airway obliteration. In our transgenic mice model, antibodies are directed exclusively against an epithelium-specific antigen. Although cytotoxic in vitro, these antibodies didn't cause severe loss of epithelium (chapter 3). In immunized mice with higher levels of epithelium-specific antibodies before transplantation (chapter 4) the epithelium injury was significantly increased. Yet, no severe OAD developed in the transplanted tracheas at 1, 3 and 6 weeks after transplantation.

Our results show that the presence of cytotoxic antibodies is insufficient to cause severe epithelial damage with subsequent OAD. Therefore it seems logical to assume that in trachea transplant models T cell responses are also involved in the development of OAD.

**Cellular responses in OAD**

It has been shown that both CD4+ and CD8+ T cells play an important role in obliteration of trachea transplants (11;30). In allograft recipients, significant numbers of pre-existing CD4+ and CD8+ T cells are able to directly recognize alloantigens (11). Indirect recognition through MHC Class II pathway mediated by CD4+ T cells and direct recognition through MHC Class I pathway mediated by CD8+ T cells are suggested to be the main mechanisms of cellular immune response (chapter 3, (31). Particularly, CD8+ T cells, namely the cytotoxic T cells (CTL) are the effector cells directly activated by class I
molecules and are shown to be mainly responsible for inducing OAD in murine trachea transplant models (32;33). This has been confirmed by T cell-adoptive transfer model (32), where transferred alloreactive CD8+ T cells could efficiently induce epithelium loss leading to the occurrence of OAD.

In our rat and mouse trachea allografts, cellular infiltration by CD4+ and CD8+ T cells started from 10 days after transplantation, indicating an acute rejection of the tracheas (chapters 2 and 3). In the hEGP-2 transgenic grafts low numbers of CD8+ T cells were induced: the CD8+/CD4+ ratio was lower than in the allografts. This may indicate that the CTL response was weak in our transgenic model compared to allotransplantation, and was not capable of inducing severe epithelium damage. This weak cellular response (in combination with the antibody response) resulted in a mild form of OAD without total obliteration of the lumen (chapters 3 and 4).

Further indication of the role of cellular responses in the development of OAD is the effect of the blocking of T cell responses by anti-CD45RB antibodies (chapter 5). This treatment reduced the level of epithelium injury from heavy loss (allografts without antibody treatment) to only abnormality (flattened epithelium in antibody treated grafts) and thus prevented OAD developed (chapter 5). Other studies diminishing T cell responses by immunosuppressive agents (34;35) or T cell depletion show the same effect on protecting epithelium and preventing OAD. These data suggest that the T cell responses contribute to epithelium injury, inducing the development of OAD.

We conclude that antibody responses together with cellular responses cause OAD in trachea transplants. Our transgenic transplantation model allows for the first time to isolate the immune responses against airway epithelium. The studies with this model show that the immune responses may make the epithelium abnormal but that this is not enough to cause OAD.

**OAD is a result from epithelium loss**

We think that OAD is only found in trachea transplants after complete loss of epithelium, as shown in our studies (chapter 2, 3) and other studies (8-10). In isografts, epithelium is mildly injured by the transplantation procedure without loss and epithelium abnormality persists for only a short term (chapter 2, 5). These isografts usually recover without obliteration. A similar process is found in the hEGP-2 transgenic mice transplantation model: mild injury and abnormality with minimal loss of epithelium, resulting in minimal obliteration in
the grafts (chapter 3, 4). In fact, this minimal obliteration was caused by submucosa thickening as a result of massive immune cell infiltrates rather than by fibroproliferation. This is in sharp contrast to the severe OAD as seen in allotransplants, where severe loss of epithelium correlates with fibroproliferation and obliteration of the lumen. Furthermore, OAD is also found in the absence of rejection after removal of epithelium (denudation) from trachea isografts (chapter 2(8)). This indicates that epithelium loss is the primary cause of fibroproliferation. Both in isografts and allografts, epithelium injury induces a general process in which injury and repair are balanced to a certain point. When the injury causes only a mild degree of epithelium injury (as we observed in the isografts and hEGP-2 grafts) the epithelium regeneration will be the dominant process. Thus, the integrity of luminal epithelium coverage can be maintained and fibroproliferation is not activated. Once the injury causes severe epithelium damage (as we observed in allografts), the epithelium regeneration process can not sufficiently maintain the epithelium integrity, thus fibroproliferation as alternative repairing process is activated. This will eventually manifest as OAD. In this concept we think that obliteration of airways can be prevented as long as protection of the epithelium is sufficient to avoid severe loss of epithelial cells.

**Epithelium protection and regeneration**

Different approaches to prevent OB/OAD have been tried in different centers. As airway epithelium injury is a result from alloimmune responses in lung transplantation, immunosuppressive agents such as Cyclosporin A and FK-506 are widely used to suppress immune responses and prolong graft survival (20;36-40). The principles of using these drugs are quite similar: suppressing cellular immunity. Common side effects of these drugs are nephrotoxicity and non-specific suppression of the immune system. In this way, the risk for infections and tumors increases.

For Cyclosporin A it has been proposed that it has an effect on epithelial cells: *In vitro* studies indicate that Cyclosporin A stimulates epithelial cells to produce cytokines that enhance fibroproliferation. (38;41). Therefore, new agents or methods to sufficiently suppress immune responses but avoid side effects are needed. In chapter 5 we used anti-CD45RB monoclonal antibodies (mAb) to modulate T cell response (42), protect airway epithelium, and prevent OAD. In this study we demonstrated the protective effect of anti-CD45RB mAb treatment on donor airway epithelium and its subsequent effect
in OAD prevention. Treatment of trachea recipients with anti-CD45RB mAb resulted in diminished T cell infiltration, a preserved luminal epithelium coverage and inhibition of luminal obliteration. Using the trachea transplantation model, we showed an alternative way for airway epithelium protection with specific T cells suppression for OAD prevention.

Another approach to prevent development of OAD would be the protection of airway epithelial cells by activation of ‘protective genes’ such as HO-1 (43) known to be capable to reduce local inflammation at the epithelial level in case of allograft reactivity. This might be achieved by exposure of donor lung to carbon monoxide, a treatment that was effective in a rat lung transplantation model (43). This treatment is worth further investigation on how applicable it is in clinical situation to protect epithelium.

Apart from protection, restoration of the epithelium after loss of its integrity has been shown to be effective in OAD prevention. The seeding of viable epithelial cells in denuded airways has successfully regenerated the epithelium and has prevented OAD (chapter 2) (8). Also, the reparation of damaged epithelium by recipient cells has been shown to be capable of OAD inhibition in transplanted allograft.

Although it is not evidential (44), stem cells may play a role in epithelium repair. In kidney transplantation model, stem cells were reported to re-build the kidney epithelial cells (45). A pilot study about bone-marrow derived stem cells in lung injury model suggested that bone-marrow stem cells could differentiate into epithelial cells repairing lung function. (46). The use of donor or recipient stem cells might be a future perspective for development of epithelium regenerative treatment.

To prevent injury during the clinical lung transplantation procedure, extra precautions in the clinical handling techniques, ranging from donor procedures to surgical transplantation, should be taken regarding the epithelium protection. During the period of brain death of the lung donor, inflammatory cytokines are released which are harmful to epithelial cells and cause injury (47). Inflation of the donor lung with oxygen before storing into preservation should be strictly according to the protocol; over-inflation should be avoided because high pressure may damage epithelium integrity. Furthermore, the airway should be kept clean and free from any toxic chemical fluids that may cause epithelium injury.
Remarks on animal OAD study: advantages and limitations

All animal OAD study just as in other animal studies, share the benefit of short investigation time, large in experimental animal numbers and cheap in cost. In transplant models, further benefits such as inbred strain availability for immune system study, transgenic possibility and tissue engineering, allow insight research for certain single aspect in a set up without encountering the clinical complexity. Especially for OB study, clinical OB is defined mainly by symptoms and confirmed by biopsies (20;21;36;48-54) which means every patient is different from another. It is difficult to find a group of similar OB patient that share the cause and stage in OB progress for a study. Also, the patient-tailored clinical treatment against OB might complicate the situation. These difficulties could be avoided in animal OAD models. Studies may be available in animal OAD models, in which a treatment can be standardized in a way that is impossible to test in patients. For example, to investigate the effect of immunosuppressive agents, treated groups can be compared with untreated groups. In transgenic animal models, the influence of a specific gene can be investigated for its behavior under certain circumstances (chapter 3, 21). Without animal models, we could not develop our scientific knowledge of the mechanism of human diseases.

The limitations of using animals for human disease study are also obvious. Simplest to say, they are not human. Animals and humans may have much similarity in body structure, physiology and pathology, yet, the differences between species in pathogenesis and pathophysiology may result in discordant processes. A limitation of the animal OAD model is that its pathogenesis seems to differ from that of OB. The process of the development of OB, for instance, may take several years in human lung transplants while in murine OAD models it takes only weeks or months. The use of such a model in animal experiments may lead to incorrect conclusions and to controversy in understanding of human diseases.

Another critical difference is that animals do not ‘complain’ as human does. Patients can describe the disease history and symptoms, which helps clinicians to make diagnose. In the clinical situation, OB develops gradually and the complaints of patients may lead to an early diagnosis. In an animal OAD model, the investigation time points are assigned arbitrarily and may introduce error in judgment. Thus the conclusions and the understandings based on animal model should be carefully interpreted into humans.
Possible implications from this study for clinical OB management

Our animal research is aimed to benefit clinical treatment. In general, the work in this thesis shows that airway epithelium is playing an important role in animal OAD and that protection of airway epithelium is crucial in preventing OAD. Based on the findings of the four studies in this thesis, the following suggestions might be useful for the management of lung transplantation patients.

Before and after transplantation, the induction of self-protective gene expression (HO-1, (43)) may help to preserve the integrity of the donor lung epithelium. This may be done by inhalation of a safe dose of CO, as has been discussed in chapters 2 and 3. It has been successfully used in animal model for airway protection (55).

The specific blocking of CTL response using anti-CD45RB, as described in chapter 5, may be applicable in clinical OB treatment. It is an increasingly used antibody in other organ transplantations (56). It could be also used combining other immunosuppressive drugs to reduce their dose and non-specific toxicity towards normal immune cells.

Although we did not show a severe destructive effect of antibodies towards donor epithelium, we believe it is still helpful to block its function, such as by blocking the complement binding pathway as discussed in chapter 4.

Further prospective is based on the epithelial cells seeding study. As it is difficult to envision how this is applicable in clinical situation now, but the stem cells transplant research widened the possibility for reconstruction/regeneration of the injured epithelium. Investigations on this direction may be promising on rebuilding the injured epithelium after transplantation or even already before transplantation.
Reference


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